Filamentous Bacteriophage as a Novel Therapeutic Tool for Alzheimer’s Disease Treatment

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Abstract. Antibodies towards the N-terminal region of the amyloid-β peptide (AβP) bind to Aβ fibrils, leading to their disaggregation. We developed an immunization procedure using filamentous phages displaying the only four amino acids EFRH encompassing amino acids 3–6 of the 42 residues of AβP, found to be the main regulatory site for Aβ formation. Phages displaying EFRH epitope are effective in eliciting humoral response against AβP which, in turn, relieves amyloid burden in brains of amyloid-β protein precursor transgenic mice, improving their ability to perform cognitive tasks. In order to overcome the low permeability of the blood brain barrier for targeting ‘anti-aggregating’ monoclonal antibodies (mAbs) to Aβ plaques in the brain, we applied antibody engineering methods to minimize the size of mAbs while maintaining their biological activity. Single-chain antibodies displayed on the surface of filamentous phage showed the ability to enter the central nervous system (CNS). The genetically engineered filamentous bacteriophage proved to be an efficient, nontoxic viral delivery vector to the brain, offering an obvious advantage over other mammalian vectors. The feasibility of these novel strategies for production and targeting of anti-aggregating antibodies against Aβ plaques to disease affected regions in the CNS may have clinical potential for treatment of Alzheimer’s disease.

Keywords: Alzheimer’s disease, amyloid plaques, brain delivery vector, filamentous phage, immunotherapy, single chain antibodies

INTRODUCTION

Bacteriophages are the most numerous life forms on earth. They can be found almost everywhere, from the ocean depths to hot springs, and can be isolated from soil and water as well as from the human or animal body (e.g., saliva, feces, and skin). Bacteriophages are known to be very common in the gastrointestinal tract and, together with their bacterial hosts, are an important component of gut flora [1,6,22]. All these facts reveal that mammalian organisms are very frequently exposed to interactions with bacteriophages and that these natural contacts are not incidental, but rather constant and intensive.

In recent years, it has been recognized that bacteriophages have several potential applications in the modern biotechnology industry; they have been proposed as delivery vehicles for protein and DNA vaccines, as gene therapy delivery vehicles, as alternatives to antibiotics, for the detection of pathogenic bacteria, and as tools for screening libraries of proteins, peptides or antibodies (for review, see [5]). These diversities and their ease of manipulation and production suggest the potential use in research, therapeutics and manufacturing in both the biotechnology and medical fields.

Filamentous phages, such as M13, f1 or fd, are well understood at both structural and genetic levels. They are bacterial viruses that consist of a circular...
Fig. 1. Schematic illustration of filamentous phage. The particle has 6–7 nm in diameter and ~800–2000 nm in length. The major coat protein, pVIII, is shown as small cylinders, as well as other minor proteins. Protein III can display scFv, while protein VIII can display small peptides, such as EFRH, without interfering with the life cycle of the phage.

single-stranded DNA (ssDNA) molecule, encapsulated in a protein envelope forming a rod-shaped cylindrical structure [18]. Two of the coat proteins – the major protein encoded by gene VIII (variously named pVIII) and the minor protein encoded by gene III – have surface-exposed N-terminal domains that tolerate foreign peptide inserts (see Fig. 1).

Filamentous bacteriophages are excellent vehicles for the expression and presentation of foreign peptides in a variety of biological systems [20,28]. Large repertoires of peptides and antibody fragments are displayed on their surface by cloning random oligonucleotides at the 5’-end of the genes closing for the phage coat proteins, both to minor protein pIII (gene III) and to major coat protein pVIII (gene VIII). The recombinant filamentous phage approach for obtaining specific peptide antigens has a major advantage over chemical synthesis, as the products obtained are the result of the biological fidelity of translational machinery and are not subject to the 70–94% purity levels common in the solid-phase synthesis of peptides.

In vivo administration of filamentous phages induces a strong immunological response to the phage proteins pIII and pVIII in all animals tested without any evidence of toxic effects [8]. The display of short immunogenic determinants fused to the phage surface provides the basis for the development of novel peptide vaccines [11, 21,31].

ACTIVE IMMUNIZATION AGAINST AMYLOID-β USING PHAGE-EFRH

We have previously shown that the EFRH residues located at positions 3-6 of the N terminus of amyloid-β peptide (AβP) represent the epitope of anti-aggregating mAbs within AβP [7]. The interaction of this epitope with such specific antibodies interferes with pathological effects in the central nervous system (CNS), such as inflammatory events and other pathogenic mechanisms in Alzheimer’s disease (AD) [3,4].

The immunomodulatory effect of the phage on the immune response against AβP is dependent on the number of epitopes displayed on the phage. Immunization with the EFRH phage may, in a short period (a few weeks), raise antibodies which recognize whole AβP, either if the epitope is displayed on pIII (10 copies) and/or pVIII on the phage (300 copies). The titer of such antibodies is proportional with the number of copies of EFRH on phage [17].

We used engineered filamentous phage displaying increasing numbers of EFRH epitopes as antigens and examined the effect of the immune response against AβP in terms of improving cognitive functions of treated mice [9,17], as well as alleviation of the pathology of AD.

Experiments were performed on transgenic mice which express human amyloid-β protein precursor (AβPP) as a model of AD. The human APP (hAPP) gene carries only the London (717) mutation [9] and/or both the London (717) and Swedish (670/671) mutations, resulting in an age-dependent increase in Aβ [17].

Transgenic mice model of AD were immunized with phage displaying various numbers of EFRH sequences by intraperitoneal (ip) administration. Specific anti-Aβ reactivity was obtained at the end of the immunization period. Titer levels, although moderate, were positively correlated to the copy number of EFRH epitopes displayed by the phage antigens. Titers obtained from sera of non-transgenic mice or mice that were injected only with PBS were at background levels.

Immunization with a filamentous phage carrying about 300 copies of the EFRH epitope elicited highest titers of antibodies against AβP. These antibodies are operationally similar in their in vitro and in vivo anti-aggregating properties [8,9,17,26] to monoclonal antibodies against the N-terminal region of AβP [3,4]. Cognitive functions of the animals, evaluated by testing the spatial and temporal navigation of each in the Morris Water Maze (MWM), showed considerable
improvement compared to untreated mice. Mice with relatively high levels of antibodies to EFRH behaved similarly to non-transgenic mice in the MWM test. The Aβ plaque burden was reduced in immunized hAPP transgenic mice, suggesting a dose-response relationship between the number of EFRH copies presented on the phage and reduced amyloid burden. Aβ content was measured and a dose-response relationship between the number of EFRH copies presented on the phage and the reduced Aβ peptide 1-40/42 was obtained [17]. In spite of the fact that plaque load is the most widely used pathological outcome measured in the preclinical assessment of anti-Aβ treatments, only a moderate correlation between amyloid burden and improvement in water maze performance (path length) was found [17].

In Table 1, we compare the performance of phage-EFRH immunization compared to other immunotherapies using fragments of and/or whole Aβ/β.

2. The key role of the EFRH epitope in Aβ formation and its high immunogenicity leads to anti-aggregating antibodies which recognize whole Aβ peptide, and can substitute for whole toxic fibrillar Aβ.

**FILAMENTOUS PHAGE AS A VECTOR MEDIATING DRUG DELIVERY TO THE BRAIN**

We demonstrate that the linear structure of the filamentous phage enables penetration to the brain via intranasal administration through the olfactory tract, conferring to the phage properties as a delivery vector of drugs [10].

Electron microscopy of negatively stained wild-type phage confirmed their linear structure. The correlation between the high permeability of the filamentous phage and its linear structure was measured after transfer of the phage through three lower orders of molecular mass cut-off membrane of 3 kDa (filamentous phage molecular mass is about several thousand kilodaltons). After 2 min of chloroform treatment, phage formed a spheroid structure. Filtration experiment showed slight reactivity of anti-phage in filtrate of chloroform-treated phage, but the majority of the phages were detected on the membrane, despite the fact that removed proteins might penetrate the membrane [10]. To investigate the ability
of the filamentous phage to enter the central nervous system, BALB/C female mice were challenged with single doses of \(10^{11}\) filamentous phage particles via the intranasal route (of the 10 mice immunized with wild-type phage, five received a single dose and the other five three daily doses. Another 10 mice were immunized with spheroid phages in the same distribution).

We showed a direct correlation between the number of applications and the amount of phage detected in the brain in both regions. The linear structure of the phage is suggested to confer penetration properties via various membranes. In a control experiment, we performed intranasal administration of chloroform treated spheroid phages in the mice under the same experimental conditions, and no presence of phages was detected [10, 27].

To demonstrate targeting of antibodies to the \(\alpha\beta\) plaques via phage as a delivery vector, we showed that anti-\(\alpha\beta\) ScFv displayed on the phage surface decorated \(\alpha\beta\) in vivo in APP transgenic mice exposed to intranasal administration. \(\alpha\beta\) were visualized both by ThS and fluorescent labeled anti-phage antibodies. \(\alpha\beta\) brain plaques were specifically labeled in the two brain sections (olfactory and hippocampus) where most of the early \(\alpha\beta\) is located after intranasal phage administration of ScFv in these mice. The filamentous phage maintains the biological activity of displayed foreign molecule of anti-\(\alpha\beta\) ScFv, and efficiently penetrates biological membranes, strongly indicating that the olfactory route may target the plaques into specific regions [10]. These findings confirmed that filamentous bacteriophage exhibits penetration properties to the CNS, and the ability to carry foreign molecules to target brain regions.

To conclude, genetically engineered filamentous bacteriophage proved to be an efficient and non-toxic viral delivery vector to the brain exhibiting penetration properties to the CNS, offering an obvious advantage over other mammalian vectors. The bacteriophage lacks the ability to infect mammalian cells unless designed to do so. Due to its structure, the filamentous phage is highly permeable to different kinds of membranes and, following the olfactory tract, it may directly target affected sites in the brain.

**PASSIVE IMMUNIZATION OF TRANSGENIC MICE WITH PHAGE-ScFv AGAINST AMYLOID-\(\beta\) PEPTIDE**

The ability of anti-\(\alpha\beta\) ScFv-phage to dissolve \(\alpha\beta\) plaques in vivo was demonstrated by repeated intranasal administration to hAPP transgenic mice [26,27]. The experiments were performed on transgenic mice carrying the hAPP gene with both London (717) and Swedish (670/671) mutations. The nine to ten month-old transgenic mice were treated with ScFv phage. Administrations of 10 \(\mu\)L containing \(10^{11}\) phages per mouse took place every 3 weeks for a total period of 6 months. Following passive immunizations protocol, the mice were subjected to training for the MWM for 4 days. The cognitive average of the treated animals was found to be close to that of the non-transgenic animals, indicating a healthy pattern of learning and memorizing of the new information [26, 27].

Intranasal treatment for six months with phage anti-\(\alpha\beta\) ScFv of transgenic mice overexpressing hAPP resulted in reduction of plaque load and considerable reduction of brain inflammation. Thioflavin-S staining of mice-brain sections showed that plaque load (defined as the area occupied by amyloid plaques divided by the total brain-section area) in the treated mice was an average 50% of the plaque load in the control Tg mice [26, 27]. Repeated applications of phage-ScFv demonstrate the ability of single-chain antibodies to dissolve \(\alpha\beta\) aggregates and to clear their deposits from the brain, indicating that alternative mechanisms beside Fc-mediated phagocytosis by microglia are involved in this clearance, as was suggested previously [2]. Clearance of phage was evaluated by immunohistochemistry (IHC) and titering. Most organs were IHC-negative for phage within 3 days. Virtually all were negative by 3 weeks. The half-life of phage in plasma was 3.6 hours. After 72 hours, phage was cleared from the blood, mainly through hepatic and renal excretion [33].

Several hypotheses may be considered regarding the disappearance of filamentous phage from the brain without inducing a toxic effect, as shown in histology studies, as well as the long life-span of challenged animals. As in other reported cases, immune mechanisms may be involved that activate scavenger cells as microglia [15].

ScFv antibodies delivered by intranasal application are more likely to reach the brain and exert their effect there, rather than reaching and affecting peripheral tissues. Intranasal administration was chosen as a direct delivery route to the CNS via the olfactory system. A direct extracellular pathway between the nasal passages and the brain was conclusively demonstrated previously using horseradish peroxidase (HRP), a 40 kDa protein tracer [13,14,29]. This effect on the brain of substances administered by intranasal application was previously demonstrated after administration of insulin.
intransnally to humans, showing CNS effects, while the blood glucose and serum insulin levels of treated people were not affected.

The feasibility of these novel strategies, using filamentous phages for the production and targeting of anti-aggregating antibodies against A/β plaques to disease affected regions in the CNS, may have clinical potential for treatment of AD, but, however, require further investigation.

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References


